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TITLE OF THE INVENTION (280 characters max)

INSULATED HERPESVIRUS-DERIVED GENE EXPRESSION CASSETTE FOR SUSTAINED AND REGULATABLE GENE EXPRESSION

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PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

FOR

INSULATED HERPESVIRUS-DERIVED GENE EXPRESSION CASSETTE FOR SUSTAINED AND REGULATABLE GENE EXPRESSION

BY

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AND

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Mark D. Moore

1.0 BACKGROUND OF THE INVENTION

The United States government has certain rights in the present invention pursuant to NIAID grant R01-AI48633 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and virology, and in particular, to genetic expression cassettes, and vector comprising them useful for the delivery of nucleic acid segments encoding selected therapeutic constructs (including for example, peptides, polypeptides, ribozymes, and catalytic RNA molecules), to selected cells and tissues of vertebrate animals. In particular, these genetic constructs are useful in the development of gene therapy vectors, including for example, HSV, AV, and rAAV vectors, for the treatment of mammalian, and in particular, human diseases, disorders, and dysfunctions. The disclosed compositions may be utilized in a variety of investigative, diagnostic and therapeutic regimens, including the prevention and treatment of a variety of human diseases. Methods and compositions are provided for preparing viral vector compositions comprising these genetic expression cassettes for use in the preparation of medicaments useful in central and targeted gene therapy of diseases, disorders, and dysfunctions in an animal, and in humans in particular.

1.2 DESCRIPTION OF THE RELATED ART

Currently, viral vectors show the greatest efficiency in gene transfer (reviewed in Anderson, 1998; Verma and Somia, Nature, 1997). For correction of genetic diseases such that persistent gene expression is required, herpesvirus, retrovirus, lentivirus, adenovirus, or AAV based vectors are desirable due to the integrating nature of the viral life cycle.

In considering transgene expression, there are many known situations where a transferred gene(s) is capable of a short period of expression however followed by a decline

to undetectable levels without the loss of the expression construct. These expression constructs may sustain transgene expression for periods of time up to 2 weeks and on rare occasions 2 months (Palmer *et al.*, 2000). Unfortunately, despite claims of sustained expression up to 2 months, the over-ruling factor is that one can anticipate an eventual decline of transcript levels often to near zero levels. As a result, this presents an additional variable to transgene expression; the predictability or probability of transgene expression. For the purposes of gene therapy, transgene expression kinetics must be predictable to achieve safe and reliable therapeutic effects.

The mechanisms responsible for transcript loss have been attributed to elaborate defense mechanisms used by eukaryotic cells to protect both the structure of their genomes and to oppose expression of abnormal transcription units (Bestor, 2000). These mechanisms include, but are not limited to, DNA methylation, multi-copy repeat-induced transgene silencing, post-transcriptional gene silencing (PTGS) mediated by RNAi, position effects that impose histone methylation/deacetylation. These host defense mechanisms represent a formidable barrier to many forms of gene therapy. Current gene therapy applications often depend on a construct or recombinant virus with the ability to express an agent of interest (protein or RNA) in a particular tissue. However, cells can detect alterations within their genome due to multi-copy transgene insertions or to abnormal transcripts and elicit a strong and heritable silencing effect. A common example of multi-copy transgene silencing is in the generation of transgenic animals. It had previously been found that transgene copy number was inversely proportional to the level of gene expression in some lines of transgenic mice. It is thought that end-to-end ligation of the expression construct and/or homologous recombination between construct molecules generates transgene concatemers (often 5-50 copies) that integrate at a single site within the genome (Dobie *et al.*, 1997). Unfortunately, the tandem repeats appear to contribute to a phenomenon similar to position effect varigation (PEV). PEV may be the result of position-dependent inactivation of the

expression construct mediated by the surrounding heterochromatin environment and results in the heritable maintenance of the transcription "off" state (Dobie *et al.*, 1997).

2.0 SUMMARY OF THE INVENTION

The present invention overcomes limitations inherent in the prior art by providing genetic constructs comprising nucleic acid sequences derived from Herpes Simplex Virus type I (HSV-I) that are capable of facilitating persistent/long-term and regulatable transgene expression in selected host cells. An important feature of these new gene expression cassettes is that the cassette is bounded by control elements that protect and insulate the gene expression portion of the cassette from the influence of DNA and chromatin structure that lie outside of the cassette, when the cassette is inserted into a viral vector or a cellular genome. These control elements effectively maintain the expression cassette in an accessible and transcriptionally-responsive conformation. The expression cassettes of the present invention facilitate predictable and sustained expression of a transgene regardless of where the cassette was inserted. For example, the cassette may be used to insert a transgene into a viral vector (including, for example, but not limited to adenovirus, adeno-associated virus (AAV), retrovirus (Lentivirus), or Herpesviruses), or into the genome of a eukaryotic cell, including mammalian cells such as human cells.

Following appropriate delivery or insertion of the genetic constructs into suitable recipient cells, the cassette is specifically engineered to express a gene of interest in a regulated manner for the duration of the cell's life. Importantly, this invention addresses a common and presently intractable problem associated with the failure of many gene therapy vectors or transgenic animals to express genes at predictable and sustained levels due to the repressive effects of the surrounding chromatin.

Another important aspect of the present invention is that by employing selected control elements within the genetic constructs that contain particular nucleic acid sequences,

it is possible to confer cell-type specific expression. For example, in an illustrative embodiment, the expression cassette may contain the components from HSV-1 that allow regulation of the control elements in neurons. By modifying these elements, however, one may alter the cell type and tissue specificity to allow the cassette to function in other cell types such as, for example, in the liver or in lung tissue.

In one embodiment, the cassette employs a defective form of HSV-1 vector as the vehicle to carry the gene expression cassette for *ex vivo* gene transfer to the central and peripheral nervous systems. This illustrative delivery system comprises two parts: (1) the insulated gene expression cassette and (2) a defective HSV-I based virus vector to deliver the transgene to the CNS. The ability of this cassette to maintain persistent, long-term gene expression, in a highly regulated manner, represents a powerful tool in the fields of gene therapy, basic gene expression assays, and in the development of animal disease models.

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

FIG. 1 shows an illustrative gene expression cassette of the present invention. The therapeutic gene of interest may be cloned into the multiple cloning site 3' of the LAT enhancer, while the MCS upstream of the LAT promoter may be utilized to facilitate introduction of one or more additional promoter elements for expression of the selected gene of interest.

FIG. 2 shows another an illustrative gene expression cassette of the present invention. The therapeutic gene of interest may be cloned into the multiple cloning site 3'

of the LAT enhancer, while the MCS upstream of the LAT promoter may be utilized to facilitate introduction of one or more additional promoter elements for expression of the selected gene of interest.

4.0 DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

The present invention provides genetic compositions and methods to facilitate sustained administration of one or more therapeutic agents in a regulatable fashion to selected cells and tissues within a mammal, including for example, the human central nervous system. These compositions also prolong general mammalian gene expression, and provide methods for generating animal models of human disease.

The present invention relates to a eukaryotic/mammalian gene expression cassette. Due to novel insulator/boundary elements, the expression cassette can be used for directing permanent regulatable expression of heterologous genes in eukaryotic cells. As such it could be applied to viral vectors for gene delivery, direct gene therapy, transgenic animals, and the development of animal disease models.

Key elements of this invention are derived from Herpes Simplex Virus Type 1 (HSV-1). Herpesviruses possess a unique neurotropic lifestyle characterized by their ability

to remain latent in neurons for the lifetime of the infected host cell. The herpes simplex virus type I is an example of the Alphaherpesvirus subfamily that has evolved a unique lifestyle that permits lytic infection in some cell types and the establishment of latency within neurons. Throughout latency, the circularized genome is maintained as a stable nucleosomal episome. Unlike lytic phase transcription, the latent phase transcriptional profile is characterized by the expression of one transcript, the latency-associated transcript (LAT), while the remainder of the genome remains largely transcriptionally silent.

The LAT locus maps to two inverted long repeat units that compose <12% of the total genome. Although this represents an overall small investment in genetic information, it is clear that the LAT locus represents an evolutionarily crucial adaptation required for the viral life-cycle. Aside from LAT, several key immediate-early genes that promote lytic phase transcription also map within this region, although they remain transcriptionally repressed during latency. This extraordinary ability of the LAT locus to escape transcriptional repression suggested that this locus was transcriptionally-privileged and insulated from the repressive effects of the surrounding genome despite its proximity to repressed lytic-phase genes. It has been recently demonstrated that the basis of this region's ability to escape transcriptional repression is at the level of chromatin structure. This unique characteristic further suggested that with suitable development, components of this region may be exploited in the construction of expression cassette(s) that are capable of facilitating persistent/permanent regulatable gene expression. With modification, these novel insulator/boundary elements provide a useful tool for the development of transgenic animals devoid of PEV in addition to the development of constructs for gene therapy, vaccine production, and methods of assaying for gene function.

4.1 GENE THERAPY VECTORS

The field of gene therapy offers a promising therapeutic strategy for the treatment of a wide variety of human diseases of the central nervous system including Alzheimer's, Parkinson's, Huntington's Diseases and Fragile-X Mental Retardation Syndrome as examples. Many chronic and progressive diseases require sustained or regulatable administration of the therapeutic gene to achieve successful treatment. Unfortunately, progress *via* conventional gene therapy has been slow as a result of transgene down-regulation due to host cell silencing mechanisms. These mechanisms include, but are not limited to, histone methylation/deacetylation, DNA methylation, position effects, or transgene copy number. This has limited the usefulness of current gene therapy vector technology for developing treatments for chronic and progressive genetic disorders. This invention addresses this problem by providing a novel set of control elements that permit a gene expression cassette to be insulated from the effects of surrounding DNA, and possesses structural features that maintain a transcriptionally accessible and regulatable environment for the expression of transgenes in a number of viral and cellular systems.

In illustrative embodiments, Herpes Simplex Virus type 1 vectors may be utilized to deliver the gene expression cassettes, because they have many advantages when considering gene delivery vectors. These include the ability to package large DNA insertions. In addition, HSV-I is neurotropic and establishes life-long infection in neurons in which the genome is maintained as a stable episome. Moreover, HSV-1 maintains the ability to infect and replicate within a wide range of human cell lines with high efficiencies.

4.2 PRODUCTION OF TRANSGENIC ANIMALS

Animal models of human disease are often an invaluable asset for use in biomedical research. However, generating transgenic or knock-out animals to accurately model human disease is no trivial task. The insulated nature of the gene expression cassette provides a

way to circumvent problems, such as embryonic lethals, associated with generating these animals. For example, current methods may use *cre-lox* systems to get past embryonic lethal animals, but the gene will be knocked out in all cells. Perhaps there are alternative uses for a particular gene product in various cells. The gene expression cassettes provided by the present invention represent a new and reliable method for gene knock-out within the subset of cells corresponding directly to the cell-type specific boundary and insulation effects of the cassette. Regardless, the ability to maintain the expression cassette in an accessible and transcriptionally-responsive conformation provides the opportunity to regulate gene expression at desired times in development. In addition, the genetic expression elements of the present invention may also be applied to the production of transgenic animals that are to be used for the production of large amounts of a transgene for pharmacologic or agricultural purposes.

It is contemplated that in some instances the genome of a transgenic non-human animal of the present invention will have been altered through the stable introduction of one or more of the genetic expression elements described herein, either native, synthetically modified, or mutated. In particular, such genetic expression elements may be provided to cells of such animals using viral vectors, such as, for example, HSV, lentiviral, retroviral, AV, or rAAV vectors. As used herein, the term "transgenic animal" is intended to refer to an animal that has incorporated exogenous DNA sequences into its genome. In designing a heterologous gene for expression in animals, sequences which interfere with the efficacy of gene expression, such as polyadenylation signals, polymerase II termination sequences, hairpins, consensus splice sites and the like are eliminated. Current advances in transgenic approaches and techniques have permitted the manipulation of a variety of animal genomes *via* gene addition, gene deletion, or gene modifications (Franz *et al.*, 1997). For example, mosquitoes (Fallon, 1996), trout (Ono *et al.*, 1997), zebrafish (Caldovic and Hackett, 1995), pigs (Van Cott *et al.*, 1997) and cows (Haskell and Bowen, 1995), are just a few of the

many animals being studied by transgenics. The creation of transgenic animals that express human proteins such as α-1-antitrypsin, in sheep (Carver *et al.*, 1993); decay accelerating factor, in pigs (Cozzi *et al.*, 1997), and plasminogen activator, in goats (Ebert *et al.*, 1991) has previously been demonstrated. The transgenic synthesis of human hemoglobin (U. S. Patent 5,602,306) and fibrinogen (U. S. Patent 5,639,940) in non-human animals have also been disclosed, each specifically incorporated herein by reference in its entirety. Further, transgenic mice and rat models have recently been described as new directions to study and treat cardiovascular diseases such as hypertension in humans (Franz *et al.*, 1997; Pinto-Siestma and Paul, 1997). The construction of a transgenic mouse model has recently been used to assay potential treatments for Alzheimer's disease (U. S. Patent 5,720,936, specifically incorporated herein by reference in its entirety). It is contemplated in the present invention that transgenic animals contribute valuable information as models for studying the effects of viral vector-delivered therapeutic compositions on correcting genetic defects and treating a variety of disorders in an animal.

4.3 ADENO-ASSOCIATED VIRUS

Adeno-associated virus is a single-stranded DNA-containing, non-pathogenic human parvovirus that is being widely investigated as a therapeutic vector for a host of muscle disorders (Muzyczka, 1992; Kessler *et al.*, 1996; Clark *et al.*, 1997; Fisher *et al.*, 1997). Six serotypes of the virus (AAV1-6) were originally described, and two more have recently been identified in rhesus macaques (Gao *et al.*, 2002). Recombinant adeno-associated virus (rAAV) vectors have been developed in which the *rep* and *cap* open reading frames of the wild-type virus have been completely replaced by a therapeutic or reporter gene, retaining only the characteristic inverted terminal repeats (ITRs), the sole *cis*-acting elements required for virus packaging. Using helper plasmids expressing various combinations of the AAV2 *rep* and AAV1, 2, and 5 *cap* genes, respectively, efficient cross-packaging of AAV2

genomes into particles containing the AAV1, 2, or 5 capsid protein has been demonstrated (Grimm *et al.*, 2003; Xiao *et al.*, 1999; Zolotukhin *et al.*, 2002; Rabinowitz *et al.*, 2002). The various serotype vectors have demonstrated distinct tropisms for different tissue types *in vivo*, due in part to their putative cell surface receptors. Although several reports have indicated that rAAV1 vectors efficiently transduce skeletal muscle in general (Fraites *et al.*, 2002; Chao *et al.*, 2001; Hauck and Xiao, 2003), no study to date has reported which of the serotypes, if any, might transduce the diaphragm in particular.

4.4 PROMOTERS AND ENHANCERS

Recombinant vectors form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, to generate a therapeutic agent from a transcribed gene that is comprised within one or more of the insulated HSV-derived gene expression cassettes disclosed herein.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively linked," "operably linked," "operatively positioned," "under the control of" or "under the transcriptional control of" means that the promoter is in the correct location and orientation in relation to the nucleic acid segment that comprises the therapeutic gene to properly facilitate, control, or regulate RNA polymerase initiation and expression of the therapeutic gene to produce the therapeutic peptide, polypeptide, ribozyme, or antisense RNA molecule in the cells that comprise and express the genetic construct.

In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the therapeutic agent-encoding polynucleotide segment under the control of one or more recombinant, or heterologous, promoter(s). As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with the particular therapeutic gene of interest in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the expression of the therapeutic agent-encoding nucleic acid segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced DNA segment.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart

before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter, such as a β-actin, AAV, AV, CMV or HSV promoter. In certain aspects of the invention, inducible promoters, such as tetracycline-controlled promoters, are also contemplated to be useful in certain cell types.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 1 and 2 below list several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of the therapeutic agents that are comprised within the disclosed insulated HSV-derived gene expression constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989

PROMOTER/ENHANCER	REFERENCES
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Orntz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987a
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
$\alpha_{1\text{-Antitrypain}}$	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988

PROMOTER/ENHANCER	REFERENCES
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b

ELEMENT	INDUCER	REFERENCES
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous nucleic acid segment, such as DNA segment that leads to the transcription of a therapeutic agent, such as a therapeutic peptide, polypeptide, ribozyme, antisense, or catalytic mRNA molecule has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous polynucleotide segment. Engineered cells are thus cells having nucleic acid segment introduced through the hand of man.

To express a therapeutic gene in accordance with the present invention one would prepare an insulated HSV-derived gene expression vector that comprises at least a first sequence region that encodes a therapeutic peptide polypeptide ribozyme or antisense mRNA under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates

transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of "recombinant expression" in this context.

4.5 PHARMACEUTICAL COMPOSITIONS

In certain embodiments, the present invention concerns formulation of one or more of the insulated HSV-derived gene expression cassettes disclosed herein in pharmaceutically acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. In particular, the present invention contemplates the formulation of one or more viral vectors, virions, or virus particles (or pluralities thereof) that comprise one or more of the disclosed insulated HSV-derived gene expression cassettes.

In such pharmaceutical compositions, it will also be understood that, if desired, the encoded nucleic acid segment, RNA, DNA or PNA compositions that express one or more therapeutic gene product(s) as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, peptides, proteins or polypeptides or various pharmaceutically-active agents, including one or more systemic or topical administrations of viral vector formulations described herein. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The viral vector compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA, DNA, or PNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment

regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.,* oral, topical, sublingual, subcutaneous, transdermal, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally as described in U.S. Patent 5,543,158; U.S. Patent 5,641,515 and U.S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as freebase or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.,* glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or

sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a mammal, and in particular, when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. In certain embodiments, the compositions of the present invention may be formulated for topical, or

transdermal delivery to one or more tissue sites or cell types within the body of the vertebrate being treated. Alternatively, in the embodiments where *ex vivo* or *ex situ* modalities are preferred, the compositions of the invention my be used externally from the body of the intended recipient by first contacting a cell suspension or a tissue sample, or other extracorporeal composition with the compositions to facilitate transfer of the viral vectors into the cells or tissues in *ex vivo* fashion. Following suitable transfection, then, such cells or tissues could be reintroduced into the body of the animal being treated.

4.6 LIPOSOME-, NANOCAPSULE-, AND MICROPARTICLE-MEDIATED DELIVERY

In certain embodiments, the genetic constructs of the present invention, and/or the virus particles or virions comprising them may further comprise one or more liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for enhancing, facilitating, or increasing the effectiveness of introducing the gene therapy constructs of the present invention into suitable host cells, tissues, or organs. In particular, the addition of a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like to the compositions of the invention may serve to enhance or facilitate the delivery of the vectors, virions, or virus particles into the target cells or tissues.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the gene expression cassettes and viral vector constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically incorporated herein by reference in its entirety). Further, various methods of liposome and

liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, 1990; Muller *et al.*, 1990). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs (Heath and Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta and Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller and Baltimore, 1984), transcription factors and allosteric effectors (Nicolau and Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*, 1985a; 1985b; Coune, 1988; Sculier *et al.*, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori and Fukatsu, 1992).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the

aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1980), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and

tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be are easily made, as described (Couvreur *et al.*, 1980; Couvreur, 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

4.7 THERAPEUTIC AND DIAGNOSTIC KITS

The invention also encompasses one or more polynucleotide compositions together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular viral vector formulations, and in the preparation of therapeutic agents for administration to a mammal, and in particularly, to a human, for one or more of the indications described herein for which viral vector-based gene therapy provides an alternative to current treatment modalities. In particular, such kits may comprise one or more viral vector compositions that comprise at least a first gene expression cassette in combination with instructions for using the viral vector in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include murines, bovines, equines, porcines, canines, and felines. The composition may include partially or significantly purified gene expression cassette-comprising viral vector compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

Therapeutic kits may also be prepared that comprise at least one of the compositions disclosed herein and instructions for using the composition as a therapeutic agent. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed genetic composition(s) may be placed, and preferably suitably aliquoted. Where a second therapeutic composition is also provided, the kit may also contain a second distinct container means into which this second composition may be placed. Alternatively, the plurality of therapeutic compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container means. The kits of the present invention will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.,* injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.8 Methods of Nucleic Acid Delivery and DNA Transfection

In certain embodiments, it is contemplated that one or more of the viral vector-delivered therapeutic product-encoding RNA, DNA, PNAs and/or substituted polynucleotide compositions disclosed herein will be used to transfect an appropriate host

cell. Technology for introduction of viral vectors comprising one or more PNAs, RNAs, and DNAs into target host cells is well known to those of skill in the art.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention for use in certain *in vitro* embodiments, and under conditions where the use of viral vector-mediated delivery is less desirable. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984; Suzuki et al., 1998; Vanbever *et al.*, 1998), direct microinjection (Capecchi, 1980; Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Takakura, 1998) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990; Klein *et al.*, 1992), and receptor-mediated transfection (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

4.9 EXPRESSION IN ANIMAL CELLS

The inventors contemplate that the expression cassettes of the present invention that comprise one or more contiguous nucleic acid sequences that encodes a therapeutic agent of the present invention may be utilized to treat one or more cellular defects in a host cell that comprises the vector. Such cells are preferably animal cells, including mammalian cells such as those obtained from a human or other primates, murine, canine, feline, ovine, caprine, bovine, equine, epine, or porcine species. In particular, the use of such constructs for the treatment and/or amelioration of disorders, dysfunctions, and diseases in a human subject suspected of suffering from such a condition is highly contemplated. The cells may be transformed with one or more viral vectors comprising one or more of the disclosed

expression constructs, such that the encoded therapeutic agent is introduced into and expressed in the host cells of the animal is sufficient to alter, reduce, ameliorate or prevent the deleterious or disease conditions either *in vitro* and/or *in vivo*.

4.10 SITE-SPECIFIC MUTAGENESIS

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent polypeptides, through specific mutagenesis of the underlying polynucleotides that encode them. The technique, well-known to those of skill in the art, further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed genetic constructs to alter the activity or effectiveness of such constructs in increasing or altering their therapeutic activity, or to effect higher or more desirable introduction in a particular host cell or tissue. Likewise in certain embodiments, the inventors contemplate the mutagenesis of the therapeutic genes comprised in such viral vectors themselves, or of the viral vector delivery vehicle to facilitate improved regulation of the particular therapeutic construct's activity, solubility, stability, expression, or efficacy *in vitro, in situ,* and/or *in vivo*.

The techniques of site-specific mutagenesis are well known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector that includes within its sequence a DNA sequence that encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful

species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation that result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing. Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety.

A number of template dependent processes are available to amplify the target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety. Briefly, in PCR™, two primer sequences are prepared which are complementary to

regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase (e.g., *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction product and the process is repeated. Preferably reverse transcription and PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction (referred to as LCR), disclosed in Eur. Pat. Appl. Publ. No. 320,308 (specifically incorporated herein by reference in its entirety). In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U. S. Patent No. 4,883,750, incorporated herein by reference in its entirety, describes an alternative method of amplification similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Intl. Pat. Appl. Publ. No. PCT/US87/00880, incorporated herein by reference in its entirety, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]triphosphates in one strand of a restriction site (Walker *et al.*, 1992), may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids that involves multiple rounds of strand displacement and synthesis, *i.e.* nick translation. A similar method, called Repair Chain Reaction (RCR) is another method of amplification which may be useful in the present invention and is involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA.

Sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having a 3' and 5' sequences of non-target DNA and an internal or "middle" sequence of the target protein specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe are identified as distinctive products by generating a signal that is released after digestion. The original template is annealed to another cycling probe and the reaction is repeated. Thus, CPR involves amplifying a signal generated by hybridization of a probe to a target gene specific expressed nucleic acid.

Still other amplification methods described in Great Britain Pat. Appl. No. 2 202 328, and in PCT Intl. Pat. Appl. Publ. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template and enzyme dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an

excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS) (Kwoh *et al.*, 1989; PCT Intl. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference in its entirety), including nucleic acid sequence based amplification (NASBA) and 3SR. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer that has sequences specific to the target sequence. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat-denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target-specific primer, followed by polymerization. The double stranded DNA molecules are then multiply transcribed by a polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into DNA, and transcribed once again with a polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target-specific sequences.

Eur. Pat. Appl. Publ. No. 329,822, incorporated herein by reference in its entirety, disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a first template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in a duplex with either DNA or

RNA). The resultant ssDNA is a second template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to its template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting as a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

PCT Intl. Pat. Appl. Publ. No. WO 89/06700, incorporated herein by reference in its entirety, disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic; i.e. new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" (Frohman, 1990), and "one-sided PCR" (Ohara *et al.*, 1989) which are well-known to those of skill in the art.

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide (Wu and Dean, 1996, incorporated herein by reference in its entirety), may also be used in the amplification of DNA sequences of the present invention.

4.11 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes may be made in the structure of the gene expression cassettes, or to the viral vectors comprising them, as well as modification to the the

therapeutic agents encoded by them and still obtain functional vectors, viral particles, and virion that encode one or more therapeutic agents with desirable characteristics. As mentioned above, it is often desirable to introduce one or more mutations into a specific polynucleotide sequence. In certain circumstances, the resulting encoded polypeptide sequence is altered by this mutation, or in other cases, the sequence of the polypeptide is unchanged by one or more mutations in the encoding polynucleotide.

When it is desirable to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, second-generation molecule, the amino acid changes may be achieved by changing one or more of the codons of the encoding DNA sequence, according to Table 3.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

TABLE 3

AMINO ACIDS		CODONS				
Alanine	Ala	A	GCA	GCC	GCG	GCU
Cysteine	Cys	C	UGC	UGU		
Aspartic acid	Asp	D	GAC	GAU		
Glutamic acid	Glu	E	GAA	GAG		

AMINO ACIDS		CODONS						
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (–0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–

1.6); histidine (–3.2); glutamate (–3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101 (specifically incorporated herein by reference in its entirety), states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their

hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.12 RIBOZYMES

In certain embodiments, aspects of the invention concerns the use of the genetic expression constructs and gene expression cassettes to deliver catalytic RNA molecules (ribozymes) to selected mammalian cells and tissues to effect a reduction or elimination of expression of one or more native DNA or mRNA molecules, so as to prevent or reduce the amount of the translation product of such mRNAs. Ribozymes are biological catalysts consisting of only RNA. They promote a variety of reactions involving RNA and DNA molecules including site-specific cleavage, ligation, polymerization, and phosphoryl exchange (Cech, 1989; Cech, 1990). Ribozymes fall into three broad classes: (1) RNAse P, (2) self-splicing introns, and (3) self-cleaving viral agents. Self-cleaving agents include hepatitis delta virus and components of plant virus satellite RNAs that sever the RNA genome as part of a rolling-circle mode of replication. Because of their small size and great specificity, ribozymes have the greatest potential for biotechnical applications. The ability of ribozymes to cleave other RNA molecules at specific sites in a catalytic manner has brought them into consideration as inhibitors of viral replication or of cell proliferation and gives them potential advantage over antisense RNA. Indeed, ribozymes have already been used to cleave viral targets and oncogene products in living cells (Koizumi *et al.*, 1992; Kashani-Sabet *et al.*, 1992; Taylor and Rossi, 1991; von-Weizsacker *et al.*, 1992; Ojwang *et al.*, 1992; Stephenson and Gibson, 1991; Yu *et al.*, 1993; Xing and Whitton, 1993; Yu *et al.*, 1995; Little and Lee, 1995).

Two kinds of ribozymes have been employed widely, hairpins and hammerheads. Both catalyze sequence-specific cleavage resulting in products with a 5N hydroxyl and a 2N,3N-cyclic phosphate. Hammerhead ribozymes have been used more commonly, because they impose few restrictions on the target site. Hairpin ribozymes are more stable and, consequently, function better than hammerheads at physiologic temperature and magnesium concentrations.

A number of patents have issued describing various ribozymes and methods for designing ribozymes. See, for example, U.S. Patent Nos. 5,646,031; 5,646,020; 5,639,655; 5,093,246; 4,987,071; 5,116,742; and 5,037,746, each specifically incorporated herein by reference in its entirety. However, the ability of ribozymes to provide therapeutic benefit *in vivo* has not yet been demonstrated.

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U. S. Patent No. 5,354,855 (specifically incorporated herein by reference) reports that certain ribozymes can act as endonucleases with a sequence-specificity greater than that

of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Six basic varieties of naturally occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA

cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, 1992). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* (1992). Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz (1989), Hampel *et al.* (1990) and U. S. Patent 5,631,359 (specifically incorporated herein by reference). An example of the hepatitis δ virus motif is described by Perrotta and Been (1992); an example of the RNaseP motif is described by Guerrier-Takada *et al.* (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990; Saville and Collins, 1991; Collins and Olive, 1993); and an example of the Group I intron is described in U. S. Patent 4,987,071 (specifically incorporated herein by reference). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

In certain embodiments, it may be important to produce enzymatic cleaving agents that exhibit a high degree of specificity for the RNA of a desired target, such as one of the sequences disclosed herein. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target mRNA. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required, although in preferred

embodiments the ribozymes are expressed from DNA or RNA vectors that are delivered to specific cells.

Small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead or the hairpin structure) may also be used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. Alternatively, catalytic RNA molecules can be expressed within cells from eukaryotic promoters (*e.g.*, Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992; Dropulic *et al.*, 1992; Weerasinghe *et al.*, 1991; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Sarver *et al.*, 1990). Those skilled in the art realize that any ribozyme can be expressed in eukaryotic cells from the appropriate DNA vector. The activity of such ribozymes can be augmented by their release from the primary transcript by a second ribozyme (Int. Pat. Appl. Publ. No. WO 93/23569, and Int. Pat. Appl. Publ. No. WO 94/02595, both hereby incorporated by reference; Ohkawa *et al.*, 1992; Taira *et al.*, 1991; and Ventura *et al.*, 1993).

Ribozymes may be added directly, or can be complexed with cationic lipids, lipid complexes, packaged within liposomes, or otherwise delivered to target cells. The RNA or RNA complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, aerosol inhalation, infusion pump or stent, with or without their incorporation in biopolymers.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595 (each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Hammerhead or hairpin ribozymes may be individually analyzed by computer folding (Jaeger *et al.*, 1989) to assess whether the ribozyme sequences fold into the appropriate secondary structure, as described herein. Those ribozymes with unfavorable

intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 or so bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Ribozymes of the hammerhead or hairpin motif may be designed to anneal to various sites in the mRNA message, and can be chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.* (1987) and in Scaringe *et al.* (1990) and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5′-end, and phosphoramidites at the 3′-end. Average stepwise coupling yields are typically >98%. Hairpin ribozymes may be synthesized in two parts and annealed to reconstruct an active ribozyme (Chowrira and Burke, 1992). Ribozymes may be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2′-amino, 2′-C-allyl, 2′-flouro, 2′-o-methyl, 2′-H (for a review see *e.g.,* Usman and Cedergren, 1992). Ribozymes may be purified by gel electrophoresis using general methods or by high-pressure liquid chromatography and resuspended in water.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Perrault *et al*, 1990; Pieken *et al.*, 1991; Usman and Cedergren, 1992; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U.S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

A preferred means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990; Gao and Huang, 1993; Lieber *et al.*, 1993; Zhou *et al.*, 1990). Ribozymes expressed from such promoters can function in mammalian cells (Kashani-Sabet *et al.*, 1992; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Yu *et al.*, 1993; L'Huillier *et al.*, 1992; Lisziewicz *et al.*, 1993). Although incorporation of the present ribozyme constructs into adeno-associated viral vectors is preferred, such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, other viral DNA vectors (such as adenovirus vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular,

intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraocular, retinal, subretinal, intraperitoneal, intracerebroventricular, intrathecal delivery, and/or direct injection to one or more tissues of the brain. More detailed descriptions of ribozyme and rAAV vector delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Ribozymes and the AAV vectored-constructs of the present invention may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of one or more neural diseases, dysfunctions, cancers,, and/or disorders. In this manner, other genetic targets may be defined as important mediators of the disease. These studies lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).

4.13 ANTISENSE OLIGONUCLEOTIDES

In certain embodiments, the gene expression constructs of the invention, and the viral vectors comprising them will find utility in the delivery of one or more antisense oligonucleotides or polynucleotides for inhibiting the expression of a selected mammalian mRNA in a host cell that has been transformed with the construct.

In the art the letters, A, G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adenosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uridine (Ura). As used in the specification and claims, compounds that are "antisense" to a particular PNA, DNA or mRNA "sense" strand are nucleotide compounds that have a nucleoside sequence that is complementary to the sense strand. It will be understood by those skilled in the art that the present invention broadly includes oligonucleotide

compounds that are capable of binding to the selected DNA or mRNA sense strand. It will also be understood that mRNA includes not only the ribonucleotide sequences encoding a protein, but also regions including the 5'-untranslated region, the 3'-untranslated region, the 5'-cap region and the intron/exon junction regions.

The invention includes compounds which are not strictly antisense; the compounds of the invention also include those oligonucleotides that may have some bases that are not complementary to bases in the sense strand provided such compounds have sufficient binding affinity for the particular DNA or mRNA for which an inhibition of expression is desired. In addition, base modifications or the use of universal bases such as inosine in the oligonucleotides of the invention are contemplated within the scope of the subject invention.

The antisense compounds may have some or all of the phosphates in the nucleotides replaced by phosphorothioates (X=S) or methylphosphonates ($X=CH_3$) or other C_{1-4} alkylphosphonates. The antisense compounds optionally may be further differentiated from native DNA by replacing one or both of the free hydroxy groups of the antisense molecule with C_{1-4} alkoxy groups ($R=C_{1-4}$ alkoxy). As used herein, C_{1-4} alkyl means a branched or unbranched hydrocarbon having 1 to 4 carbon-atoms.

The disclosed antisense compounds also may be substituted at the 3' and/or 5' ends by a substituted acridine derivative. As used herein, "substituted acridine," means any acridine derivative capable of intercalating nucleotide strands such as DNA. Preferred substituted acridines are 2-methoxy-6-chloro-9-pentylaminoacridine, *N*-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-3-aminopropanol, and *N*-(6-chloro2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-5-aminopentanol. Other suitable acridine derivatives are readily apparent to persons skilled in the art. Additionally, as used herein "P(O)(O) -substituted acridine" means a phosphate covalently linked to a substitute acridine.

As used herein, the term "nucleotides" includes nucleotides in which the phosphate moiety is replaced by phosphorothioate or alkylphosphonate and the nucleotides may be substituted by substituted acridines.

In one embodiment, the antisense compounds of the invention differ from native DNA by the modification of the phosphodiester backbone to extend the life of the antisense molecule. For example, the phosphates can be replaced by phosphorothioates. The ends of the molecule may also be optimally substituted by an acridine derivative that intercalates nucleotide strands of DNA. Intl. Pat. Appl. Publ. No. WO 98/13526 and U. S. Patent 5,849,902 (each specifically incorporated herein by reference in its entirety) describe a method of preparing three component chimeric antisense compositions, and discuss many of the currently available methodologies for synthesis of substituted oligonucleotides having improved antisense characteristics and/or half-life.

The reaction scheme involves ^{1}H-tetrazole-catalyzed coupling of phosphoramidites to give phosphate intermediates that are subsequently reacted with sulfur in 2,6-lutidine to generate phosphate compounds. Oligonucleotide compounds are prepared by treating the phosphate compounds with thiophenoxide (1:2:2 thiophenol/triethylamine/tetrahydrofuran, room temperature, 1 hr). The reaction sequence is repeated until an oligonucleotide compound of the desired length has been prepared. The compounds are cleaved from the support by treating with ammonium hydroxide at room temperature for 1 hr and then are further deprotected by heating at about 50°C overnight to yield preferred antisense compounds.

Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, relative stability, and antisense compositions were selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions

of the mRNA, are those that are at or near the AUG translation initiation codon, and those sequences that were substantially complementary to 5′ regions of the mRNA. These secondary structure analyses and target site selection considerations were performed using v.4 of the OLIGO primer analysis software (Rychlik, 1997) and the BLASTN 2.0.5 algorithm software (Altschul *et al.,* 1997).

4.14 EXEMPLARY DEFINITIONS

In accordance with the present invention, polynucleotides, nucleic acid segments, nucleic acid sequences, and the like, include, but are not limited to, DNAs (including and not limited to genomic or extragenomic DNAs), genes, peptide nucleic acids (PNAs) RNAs (including, but not limited to, rRNAs, mRNAs and tRNAs), nucleosides, and suitable nucleic acid segments either obtained from native sources, chemically synthesized, modified, or otherwise prepared in whole or in part by the hand of man.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and compositions similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and compositions are described herein. For purposes of the present invention, the following terms are defined below:

A, an: In accordance with long standing patent law convention, the words "a" and "an" when used in this application, including the claims, denotes "one or more".

Expression: The combination of intracellular processes, including transcription and translation undergone by a polynucleotide such as, for example, a structural gene to synthesize the encoded peptide or polypeptide.

Promoter: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Regulatory Element: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Structural gene: A gene or sequence region that is expressed to produce an encoded peptide or polypeptide.

Transformation: A process of introducing an exogenous polynucleotide sequence (*e.g.*, a vector, a recombinant DNA or RNA molecule) into a host cell or protoplast in which that exogenous nucleic acid segment is incorporated into at least a first chromosome or is capable of autonomous replication within the transformed host cell. Transfection, electroporation, and naked nucleic acid uptake all represent examples of techniques used to transform a host cell with one or more polynucleotides.

Transformed cell: A host cell whose nucleic acid complement has been altered by the introduction of one or more exogenous polynucleotides into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell, or from the progeny or offspring of any generation of such a transformed host cell.

Vector: A nucleic acid molecule (typically comprised of DNA) capable of replication in a host cell and/or to which another nucleic acid segment can be operatively linked so as to bring about replication of the attached segment. A plasmid, cosmid, or a virus is an exemplary vector.

The terms "substantially corresponds to", "substantially homologous", or "substantial identity" as used herein denotes a characteristic of a nucleic acid or an amino acid sequence, wherein a selected nucleic acid or amino acid sequence has at least about 70 or about 75 percent sequence identity as compared to a selected reference nucleic acid or amino acid sequence. More typically, the selected sequence and the reference sequence will have at least about 76, 77, 78, 79, 80, 81, 82, 83, 84 or even 85 percent sequence identity, and more preferably at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 percent sequence

identity. More preferably still, highly homologous sequences often share greater than at least about 96, 97, 98, or 99 percent sequence identity between the selected sequence and the reference sequence to which it was compared. The percentage of sequence identity may be calculated over the entire length of the sequences to be compared, or may be calculated by excluding small deletions or additions which total less than about 25 percent or so of the chosen reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, in the case of sequence homology of two or more polynucleotide sequences, the reference sequence will typically comprise at least about 18-25 nucleotides, more typically at least about 26 to 35 nucleotides, and even more typically at least about 40, 50, 60, 70, 80, 90, or even 100 or so nucleotides. Desirably, which highly homologous fragments are desired, the extent of percent identity between the two sequences will be at least about 80%, preferably at least about 85%, and more preferably about 90% or 95% or higher, as readily determined by one or more of the sequence comparison algorithms well-known to those of skill in the art, such as e.g., the FASTA program analysis described by Pearson and Lipman (1988).

The term "naturally occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by the hand of man in a laboratory is naturally-occurring. As used herein, laboratory strains of rodents that may have been selectively bred according to classical genetics are considered naturally occurring animals.

As used herein, a "heterologous" is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter which does not naturally occur adjacent to the referenced

structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

"Transcriptional regulatory element" refers to a polynucleotide sequence that activates transcription alone or in combination with one or more other nucleic acid sequences. A transcriptional regulatory element can, for example, comprise one or more promoters, one or more response elements, one or more negative regulatory elements, and/or one or more enhancers.

As used herein, a "transcription factor recognition site" and a "transcription factor binding site" refer to a polynucleotide sequence(s) or sequence motif(s) which are identified as being sites for the sequence-specific interaction of one or more transcription factors, frequently taking the form of direct protein-DNA binding. Typically, transcription factor binding sites can be identified by DNA footprinting, gel mobility shift assays, and the like, and/or can be predicted on the basis of known consensus sequence motifs, or by other methods known to those of skill in the art.

As used herein, the term "operably linked" refers to a linkage of two or more polynucleotides or two or more nucleic acid sequences in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

"Transcriptional unit" refers to a polynucleotide sequence that comprises at least a first structural gene operably linked to at least a first *cis*-acting promoter sequence and optionally linked operably to one or more other *cis*-acting nucleic acid sequences necessary for efficient transcription of the structural gene sequences, and at least a first distal regulatory element as may be required for the appropriate tissue-specific and developmental transcription of the structural gene sequence operably positioned under the control of the promoter and/or enhancer elements, as well as any additional *cis* sequences that are necessary for efficient transcription and translation (*e.g.*, polyadenylation site(s), mRNA stability controlling sequence(s), *etc.*

The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an oligonucleotide sequence, is substantially complementary to all or a portion of the selected sequence, and thus will specifically bind to a portion of an mRNA encoding the selected sequence. As such, typically the sequences will be highly complementary to the mRNA "target" sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the complementary portion of the sequence. In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary to the sequence to which the oligonucleotide specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region of the mRNA and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product.

Substantially complementary oligonucleotide sequences will be greater than about 80 percent complementary (or '% exact-match') to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence

to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to all or a portion of the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

5.0 EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred

modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – INSULATED HERPESVIRUS-DERIVED GENE EXPRESSION CASSETTE FOR SUSTAINED AND REGULATABLE GENE EXPRESSION

This example describes the use of DNA elements derived/isolated from Herpes Simplex Virus type I (HSV-I) in the construction of a gene expression cassette capable of facilitating persistent/long-term and regulatable transgene expression. A novel and enabling feature of this invention is that the cassette is bounded by control elements that protect and insulate the gene expression portion of the cassette from the influence of DNA and chromatin structure that lie outside of the cassette, when the cassette is inserted into a viral vector, cellular, animal or human genome. These control elements effectively maintain the expression cassette in an accessible and transcriptionally-responsive conformation. This novel cassette therefore would allow predictable and sustained [permanent regulatable expression (PRE)] OR [silencing-resistant] expression of a transgene regardless of where the cassette was inserted in a viral vector or a host genome. A key feature of this expression cassette is that it prevents transcription of a gene in a viral vector or transgene from being shut down with time due to chromatin effects of the surrounding DNA. Solving this transcriptional shut-down problem greatly extends the application of existing viral vector and gene delivery technologies.

An integral part of this invention is the expression cassette (FIG. 1), and the novel and key features are the insulating elements that bound the cassette and protect the elements between them from silencing effects of the surrounding chromatin (FIG. 2). As mentioned, this cassette has applications in viral vector, transgenics and other gene delivery

applications. The initial embodiment of the invention may be examined in the context of an HSV-1 gene therapy vector construct. Note that, while in this particular embodiment will direct expression from this cassette in a neuron-specific manner, key control elements such as the promoter and enhancer could be replaced with similar elements conferring different tissue/cell-type specificities without altering the PRE properties of the insulating elements.

5.1.1 Expression Cassette-LAT Insulator/Boundary 1 (I/B 1) Element, Promoter(s), LAT enhancer, Heterologous Gene(s), LAT Insulator/Boundary 2 (I/B 2) Element (FIG. 1)

The components of the expression cassette invention consist of a LAT insulator/boundary 1 (I/B1) element, a promoter, the LAT enhancer region flanked by splice donor and splice acceptor sites, a heterologous transgene, and a LAT insulator/boundary 2 (I/B 2) element linked together in that order. The order of the constructs components serves to facilitate permanent and regulatable (in the case of inducible promoter(s)) gene expression. The term "permanent regulatable expression" is taken to mean expression of a heterologous gene(s) from the invention construct for the duration of the host-cell(s) life.

5.1.1.1 LAT Insulator/Boundary 1 (I/B 1) Element

The LAT insulator/boundary 1 (I/B 1) element is defined here as the region comprising HSV1 nucleotides 8,365-9,273 (GenBank NC 001806 : from SwaI-AatII sites), fragments or derivatives of this region, including homologous regions from other alphaherpesviruses that may confer alternative regulation, but are capable of conferring permanent regulatable expression of heterologous genes in the expression cassette comprising the invention.

5.1.1.2 Promoters

A promoter refers to any transcriptional promoter that corresponds to a region of DNA involved in binding of RNA polymerase to initiate transcription. This region of DNA

may range in size and complexity from minimal promoters to promoters including upstream activating sequences and enhancers/silencer elements. Within the context of the initial embodiment of this invention, the promoter consists of the HSV-1 latency active promoter 1 (LAP1) comprising nucleotides 117,938-118,843 (GenBank NC 001806 : from SmaI-SacII sites) or pHB22F nucleotides 1,173-2,013 (Berthomme *et al.*, 2000). This promoter allows neuronal-specific expression. Other promoters with different cell-type/tissue specificity could be employed, as well as ones capable of regulation.

5.1.1.3 LAT ENHANCER

An enhancer element refers to any *cis*-acting sequence that increases the utilization eukaryotic transcriptional promoters. Enhancers can function in either orientation and in any location (upstream or downstream) relative to the promoter. Within the context of the initial embodiment of this invention, the LAT enhancer consists of the HSV-1 sequence corresponding to the LAT 5' exon and comprises nucleotides 118,975-120,471 (GenBank NC 001806) or pHB22F nucleotides 2,050-3,546 (Berthomme *et al.*, 2000). Other enhancers with different cell/tissue-specific or expression properties could also be substituted.

5.1.1.4 HETEROLGOUS GENES

The term heterologous gene comprises any gene other than genes found present within the delivery vector encompassing the expression cassette. The term gene refers collectively to any nucleic acid sequence that is capable of being transcribed and therefore includes sequences encoding mRNA, tRNA, and rRNA. With respect to the growing field of RNAi, the sequence may be in the sense or antisense orientation to the promoter and used to inhibit a target host cell gene. On the other hand, sequences encoding mRNA may include either 5' and/or 3' untranslated regions, transcription stop signals, polyadenylation

signals, and/or downstream enhancer/silencer elements. The heterologous gene may encode a polypeptide for therapeutic use or for use in developing animal models of human disease. Additionally, the heterologous gene may encode antigenic polypeptides for use in vaccine development, the gene may encode a marker gene like green fluorescent protein, or the gene may encode polypeptides that function in the regulation of other genes.

5.1.1.5 LAT INSULATOR/BOUNDARY 2 (I/B 2) ELEMENT

The LAT insulator/boundary 2 (I/B 2) element is defined here as the region comprising HSV1 nucleotides 120,208-120,940 (GenBank NC 001806 : PCR fragment tagged with SpeI and NotI respectively), fragments or derivatives of this region, including homologous regions from other alphaherpesviruses that may confer alternative regulation, but are capable of conferring permanent regulatable expression of heterologous genes in the expression cassette comprising the invention.

6.0 REFERENCES

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising:

(a) an HSV LAT enhancer element;

(b) a first LAT insulator/boundary region operably positioned upstream of said LAT enhancer element; and

(c) a second LAT insulatory/boundary region operably positioned downstream of said LAT enhancer element.

2. The polynucleotide of claim 1, wherein said LAT enhancer element comprises a contiguous nucleotide sequence from an HSV LAT 5' exon.

3. The polynucleotide of claim 2, wherein said LAT enhancer element consists essentially of a contiguous nucleotide sequence from an HSV LAT 5' exon.

4. The polynucleotide of claim 3, wherein said LAT enhancer element consists of a contiguous nucleotide sequence from an HSV LAT 5' exon.

5. The polynucleotide of claim 1, wherein said LAT enhancer element comprises a contiguous nucleotide sequence from about nucleotide 118,975 to about nucloetide 120,471 of an HSV LAT 5' exon.

6. The polynucleotide of claim 5, wherein said LAT enhancer element consists essentially of a contiguous nucleotide sequence from about nucleotide 118,975 to about nucloetide 120,471 of an HSV LAT 5' exon.

7. The polynucleotide of claim 6, wherein said LAT enhancer element consists of a contiguous nucleotide sequence from about nucleotide 118,975 to about nucloetide 120,471 of an HSV LAT 5' exon.

8. The polynucleotide of claim 7, wherein said LAT enhancer element consists of a contiguous nucleotide sequence from nucleotide 118,975 to nucloetide 120,471 of an HSV LAT 5' exon.

9. The polynucleotide of any preceding claim, further comprising at least a first promoter region operably positioned upstream of said LAT enhancer element, and downstream of said first LAT insulator/boundary region.

10. The polynucleotide of claim 9, wherein said promoter region comprises an HSV LAP1 promoter.

11. The polynucleotide of claim 10, wherein said promoter region consists essentially of an HSV LAP1 promoter.

12. The polynucleotide of claim 11, wherein said promoter region consists of an HSV LAP1 promoter.

13. The polynucleotide of claim 10, wherein said promoter region comprises an HSV LAP1 promoter that comprises a sequence region of from about nucleotide 117,938 to about 118,843 of said HSV LAP1 promoter.

14. The polynucleotide of claim 13, wherein said promoter region comprises an HSV LAP1 promoter that consists essentially of a sequence region of from about nucleotide 117,938 to about 118,843 of said HSV LAP1 promoter.

15. The polynucleotide of claim 14, wherein said promoter region comprises an HSV LAP1 promoter that consists of a sequence region of from about nucleotide 117,938 to about 118,843 of said HSV LAP1 promoter.

16. The polynucleotide of claim 15, wherein said promoter region comprises an HSV LAP1 promoter that consists of a sequence region of from nucleotide 117,938 to 118,843 of said HSV LAP1 promoter.

17. The polynucleotide of any preceding claim, wherein said first LAT insulator/boundary region comprises a contiguous nucleotide sequence from an HSV insulator region or an HSV boundary region.

18. The polynucleotide of claim 17, wherein said first LAT insulator/boundary region comprises a contiguous nucleotide sequence from about nucleotide 8365 to about nucleotide 9273 of HSV1.

19. The polynucleotide of claim 18, wherein said first LAT insulator/boundary region consists essentially of a contiguous nucleotide sequence from about nucleotide 8365 to about nucleotide 9273 of HSV1.

20. The polynucleotide of claim 19, wherein said first LAT insulator/boundary region consists of a contiguous nucleotide sequence from about nucleotide 8365 to about nucleotide 9273 of HSV1.

21. The polynucleotide of claim 20, wherein said first LAT insulator/boundary region consists of a contiguous nucleotide sequence from nucleotide 8365 to nucleotide 9273 of HSV1.

22. The polynucleotide of any preceding claim, wherein said second LAT insulator/boundary region comprises a contiguous nucleotide sequence from an HSV insulator region or an HSV boundary region.

23. The polynucleotide of claim 22, wherein said second LAT insulator/boundary region comprises a contiguous nucleotide sequence from about nucleotide 120,208 to about nucleotide 120,940 of HSV1.

24. The polynucleotide of claim 23, wherein said second LAT insulator/boundary region consists essentially of a contiguous nucleotide sequence from about nucleotide 120,208 to about nucleotide 120,940 of HSV1.

25. The polynucleotide of claim 24, wherein said second LAT insulator/boundary region consists of a contiguous nucleotide sequence from about nucleotide 120,208 to about nucleotide 120,940 of HSV1.

26. The polynucleotide of claim 25, wherein said second LAT insulator/boundary region consists of a contiguous nucleotide sequence from nucleotide 120,208 to nucleotide 120,940 of HSV1.

27. The polynucleotide of any preceding claim, further comprising at least a first multiple cloning region operably positioned downstream of said first LAT insulator/boundary region and upstream of said LAT enhancer element.

28. The polynucleotide of claim 27, wherein said first multiple cloning region further comprises a nucleic acid sequence that encodes a promoter or an enhancer sequence that is expressed in a mammalian host cell.

29. The polynucleotide of any preceding claim, further comprising at least a second multiple cloning region operably positioned upstream of said second LAT insulator/boundary region and downstream of said LAT enhancer element.

30. The polynucleotide of claim 29, wherein said second multiple cloning region further comprises at least a first nucleic acid sequence that encodes a therapeutic agent.

31. The polynucleotide of claim 30, wherein said second multiple cloning region further comprises a nucleic acid sequence that encodes at least a first therapeutic agent

selected from the group consisting of a peptide, a polypeptide, a ribozyme, a catalytic RNA molecule, an antisense oligonucleotide, and an antisense polynucleotide.

32. The polynucleotide of claim 31, wherein said peptide or polypeptide is an antibody, a growth factor, a neurotrophic factor, a transcription factor, an anti-apoptotic factor, a proliferation factor, an enzyme, a cytotoxin, a transcription factor, an apoptotic factor, a tumor suppressor, a kinase, a cytokine, a lymphokine, or a protease.

33. The polynucleotide of claim 30, wherein said second multiple cloning region further comprises at least a second distinct nucleic acid sequence that encodes at least a second distinct therapeutic agent selected from the group consisting of a peptide, an antibody, a protein, a polypeptide, a ribozyme, a catalytic RNA molecule, an antisense oligonucleotide, and an antisense polynucleotide.

34. The polynucleotide of claim 33, wherein said catalytic RNA molecule specifically cleaves an mRNA encoding a transcription factor, an anti-apoptotic factor, a proliferation factor, a hormone receptor, a growth factor, an oncogenic peptide, or a growth factor polypeptide.

35. The polynucleotide of claim 34, wherein said catalytic RNA molecule is a hammerhead or a hairpin ribozyme.

36. The polynucleotide of any preceding claim, wherein said polynucleotide is from about 2000 to about 9000 nucleotides in length.

37. The polynucleotide of any preceding claim, wherein said polynucleotide is from about 2500 to about 8000 nucleotides in length.

38. The polynucleotide of any preceding claim, wherein said polynucleotide is from about 3000 to about 7000 nucleotides in length.

39. The polynucleotide of any preceding claim, wherein said polynucleotide is from about 3500 to about 6000 nucleotides in length.

40. A plasmid vector comprising the polynucleotide of any one of claims 1 to 39.

41. A viral vector, virion, or viral particle for transfection of mammalian cells, comprising the polynucleotide of any one of claims 1 to 39, or the plasmid of claim 40.

42. The viral vector, virion, or viral particle of claim 41, wherein said vector comprises a retroviral, adenoviral, adeno-associated viral, or a herpes viral vector.

43. The viral vector, virion, or viral particle of claim 41 or claim 42, wherein said vector is a gutless AV vector, a recombinant AAV vector, or a recombinant HSV vector.

44. A plurality of HSV or AAV particles comprising the polynucleotide of any one of claims 1 to 39.

45. A host cell comprising:

(a) the polynucleotide of any one of claims 1 to 39;

(b) the plasmid vector of claim 40;

(c) the viral vector, virion, or viral particle of any one of claims 41 to 43; or

(d) the plurality of HSV or AAV particles of claim 44.

46. The host cell of claim 45, wherein said host cell is a mammalian host cell.

47. The host cell of claim 45 or claim 46, wherein said mammalian host cell is a human host cell.

48. A composition comprising the polynucleotide of any one of claims 1 to 39, the plasmid vector of claim 40, the viral vector, virion, or viral particle of any one of claims 41 to 43, the plurality of HSV or AAV particles of claim 44, or the host cell of any one of claims 45 to47.

49. The composition of claim 48, further comprising a pharmaceutical excipient.

50. The composition of claim 48 or claim 49, further comprising a lipid, a liposome, a lipofection complex, a nanoparticle, or a nanocapsule.

51. The composition of any one of claims 48 to 50, wherein said pharmaceutical excipient is formulated for injection.

52. The composition of any one of claims 48 to 51, for use in therapy.

53. The composition of any one of claims 48 to 52, for use in the therapy of cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic

disease, liver disease, neurological disease, neurosensory dysfunction, stroke, ischemia, neuromuscular disorders, eating disorders, neurological diseases, neuroskeletal impairment or disability, Alzheimer's disease, Huntington's disease, Parkinson's disease, or pulmonary disease.

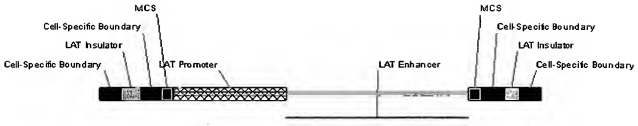
54. A kit comprising: (a) a component selected from the group consisting of the polynucleotide of any one of claims 1 to 39, the plasmid vector of claim 40, the viral vector, virion, or viral particle of any one of claims 41 to 43, the plurality of HSV or AAV particles of claim 44, the host cell of any one of claims 45 to47, or the composition of any one of claims 48-51; and (b) instructions for using said kit.

55. Use of the polynucleotide according to of any one of claims 1 to 39, in the manufacture of a medicament for treating or ameliorating the symptoms of a disease or dysfunction in a mammal.

56. Use according to claim 54 or claim 55, for use in the manufacture of a medicament for treating or ameliorating the symptoms of cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, liver disease, neurological disease, neurosensory dysfunction, stroke, ischemia, neuromuscular disorders, eating disorders, neurological diseases, neuroskeletal impairment or disability, Alzheimer's disease, Huntington's disease, Parkinson's disease, or pulmonary disease.

ABSTRACT OF THE DISCLOSURE

Disclosed are genetic expression cassettes, and vector comprising them useful for the delivery of nucleic acid segments encoding selected therapeutic constructs (including for example, peptides, polypeptides, ribozymes, and catalytic RNA molecules), to selected cells and tissues of vertebrate animals. The disclosed genetic constructs are useful in the development of gene therapy vectors, including for example, viral vectors such as HSV, retroviral, lentiviral, AV, and rAAV vectors. The expression cassettes disclosed herein provide new tools in the field of gene therapy, and for the treatment of mammalian, and in particular, human diseases, disorders, and dysfunctions. The disclosed compositions may be utilized in a variety of investigative, diagnostic and therapeutic regimens, including the prevention and treatment of a variety of human diseases.



■ HSV type I strain 17syn+ neuronal-specific DNA boundary element; Cell-type specific boundary elements may be swapped in/out

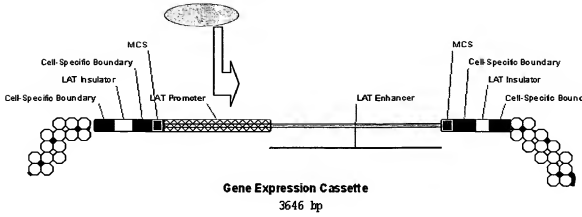
▨ HSV type I strain 17syn+ insulator element capable of protecting and maintaining the gene expression portion of the cassette in highly responsive transcriptional state

▣ Multiple cloning sites represented by a cluster of restriction enzyme sites that may be used to facilitate cloning of the gene of interest and/or an additional promoter element

⊠ HSV type I strain 17syn+ latency associated transcript (LAT) core promoter

■ HSV type I strain 17syn+ latency associated transcript (LAT) 5' exon DNA exhibiting enhancer function. The element is bound by Splice Donor (SD) and Splice Acceptor (SA) sites to facilitate splicing of the transcript's 'artificial' intron from the desired downstream gene of interest transcript. Splicing also promotes nuclear export of desired transcript.

FIG. 1



■ HSV type I strain 17syn+ neuronal-specific DNA boundary element; Cell-type specific boundary elements may be swapped in/out.

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⊠ HSV type I strain 17syn+ latency associated transcript (LAT) core promoter.

■ HSV type I strain 17syn+ latency associated transcript (LAT) 5' exon DNA exhibiting enhancer function. The element is bound by Splice Donor (SD) and Splice Acceptor (SA) sites to facilitate splicing of the transcript's 'artificial' intron from the desired downstream gene of interest transcript. Splicing also promotes nuclear export of desired transcript.

Transcriptionally repressed regions of DNA located outside of the insulated cassette.

○ RNA Polymerase easily initiating transcription within conformationally accessible expression cassette.

FIG. 2